THE ISOLATED F_0 OF *ESCHERICHIA COLI* ATP-SYNTHASE IS RECONSTITUTIVELY ACTIVE IN H^+-CONDUCTION AND ATP-DEPENDENT ENERGY-TRANSDUCTION

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1. Introduction

The membrane-bound ATP-synthases of different organisms have common structural and functional properties [1–3]. The membrane-associated part, F_1, of the enzyme bears ATPase activity, the membrane-integrated part, F_0, catalyzes H^+-conduction across the membrane. Both parts, F_1 and F_0, are necessary for energy-transducing reactions, i.e., reactions coupled with a H^+-translocation across the membrane. Binding of *N,N'*-dicyclohexylcarbodiimide to F_0 blocks the H^+-conduction [4–6] and thereby inhibits both ATP-synthesis and ATP-hydrolysis of the ATP-synthase.

The intact ATP-synthase of *Escherichia coli* has been purified and shown to consist of 8 different polypeptides [7,8]: 5 subunits of F_1 ($\alpha,\beta,\gamma,\delta,\epsilon$) and 3 likely subunits of F_0 (a,b,c). The DCCD-binding protein, c, is the only component of F_0, which has been thoroughly characterized. It is an extremely hydrophobic protein of M_r 8500 [5,9] and the amino acid sequence of protein c from wild-type and various ATP-synthase mutants is known [10–12]. There exists genetic [13] and biochemical (14) evidence, that the other 2 polypeptides, a and b, are also necessary for the expression of H^+-conduction via F_0.

The purification of an intact F_0 will help to elucidate the structure of F_0 and its components and will facilitate the functional characterization of H^+-conduction. Here we describe the purification of F_0 from *Escherichia coli*. The reconstitution of ATP-dependent H^+-translocation with F_0, F_1 and phospholipids as well as the reconstitution of H^+-conduction with F_0 and phospholipids shows that the purified F_0 is fully functional. It consists of the 3 polypeptides a, b and c.

Abbreviations: F_1F_0, ATP-synthase of oxidative phosphorylation; F_1, ATPase moiety of F_1F_0; F_0, H^+-conducting moiety of F_1F_0, DCCD, *N,N'*-dicyclohexylcarbodiimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole; SDS, sodium dodecylsulfate; PEG 6000 or 400, polyethyleneglycol of M_r 6000 or 400; EDTA, ethylenediaminetetraacetate

2. Materials and methods

ATP-synthase [7], F_1-ATPase [15] and F_1-depleted membranes [16] were prepared as described. Proteoliposomes were reconstituted as in [17] or by a modification of the dialysis method [18]: 50 μl F_1F_0-, F_0- or (F_0 + F_1)-preparations were added to 200 μl phospholipid suspension (10 mM tricine–NaOH (pH 8), 0.2 mM EDTA, 0.8% deoxycholate, 1.6% cholate, 20–30 mg asolectin/ml; 1 ml mixture sonicated for 5 min with 50–80 W, Labsonic 1510, Microtip) and the sample was dialyzed against a 1000-fold vol. buffer (10 mM tricine–KOH (pH 7.8), 2.5 mM $MgSO_4$, 50 μM $CaCl_2$) for 15–18 h at 12°C. Preparation of K^+-loaded vesicles [16], assay of H^+-conduction [16] and of ATP-dependent H^+-translocation [7] and protein determination [19] were performed as described. SDS gel electrophoresis was done as in [20] with the following modification: dimension of the gel 60 × 70 × 0.75 mm, diallyltardiamide (3.3% of total acrylamide) instead of *N,N'*-methylene-bis-acrylamide, 0.15% (w/v) SDS in all buffers and 10% (w/v) glycerol in the lower gel.

For the preparation of F_0 the ATP-synthase was concentrated in two steps.

1. Either rechromatography on DEAE–Sepharose Cl-6-B (0.25 × vol. of the first column) or addition of dry Sephadex-G-100 (8 g/100 ml eluate, 3–4 h swelling at 0°C); the resulting protein concentra-

tion of >1 mg/ml was necessary for a quantitative precipitation of F_1F_0 with PEG.

2. The enzyme solution was adjusted to ~100 mM KCl by dialysis (3 h, 0°C) against a 2-fold vol. buffer (without KCl and Aminoxid). $MgCl_2$ (10 mM) and 12.5% (w/v) PEG 6000 (from a 50% stock solution) were added and after stirring the sample at 0°C for 5 min the precipitate was collected by centrifugation (15 min, 25 000 × *g*). The enzyme was resuspended in buffer [7] at 10 mg protein/ml and stored in liquid nitrogen.

F_1F_0 was diluted to 2 mg/ml in buffer (20 mM MOPS (pH 7), 10 mM $MgCl_2$, 100 mM KCl, 12 mM taurodeoxycholate, 20% (w/v) glycerol), precipitated by addition of an equal volume of PEG 400, incubated on ice for 5 min and centrifuged at 40 000 × *g* for 10 min. The sediment was resuspended in the original volume buffer and the procedure repeated. The final pellet was resuspended in twice the original volume buffer (50 mM Tris–HCl (pH 7.8), 0.2 mM $MgCl_2$) and centrifuged for 10 min at 220 000 × *g*. The supernatant was decanted, adjusted to 1.2 mM EDTA, 1 mM dithiothreitol, 1 M KSCN, incubated on ice for 20 min and centrifuged at 220 000 × *g* for 45 min. The sediment was resuspended in buffer (50 mM Tris–HCl (pH 7.8), 1 mM $MgCl_2$, 1 mM DTE) at ~2 mg protein/ml. The preparation has a turbid, silky appearance and contains F_0 probably in a particulate form. Where indicated the treatment with DCCD was performed after the first dilution of F_1F_0 as in [7].

DCCD and PEG were purchased from Serva (Heidelberg), asolectin and taurodeoxycholate from Sigma (München), Aminoxid WS 35 from Goldschmidt (Essen), TTFB and ACMA were generous gifts from Dr Beechey (Sittingbourne) and Professor Overath (Tübingen). All other fine chemicals were purchased from Boehringer (Mannheim), all other chemicals from Merck (Darmstadt).

3. Results

We tried various agents and methods to dissociate F_1F_0 and to isolate an intact F_0. Integration of F_1F_0 into liposomes and repeated washing of proteoliposomes with EDTA buffers resulted in a preparation where the ATP-dependent H^+-translocation could be reconstituted to 90% by addition of F_1, that means, the F_1F_0-complex was dissociated to this extent. The SDS gel electrophoresis revealed that >50% of these 'F_0-vesicles' consisted of F_1 subunits, probably unspecifically adsorbed to the liposomes (cf. [21]). We did not find a way to remove the residual F_1 polypeptides. Similar results were obtained by first dissociating the enzyme with $NaClO_4$, guanidine–HCl or urea and then reconstituting proteoliposomes.

Delipidisation of a membrane-bound ATPase by precipitation with PEG in a buffer containing deoxycholate and 20% glycerol has been reported [22]. We obtained similar results with taurodeoxycholate instead

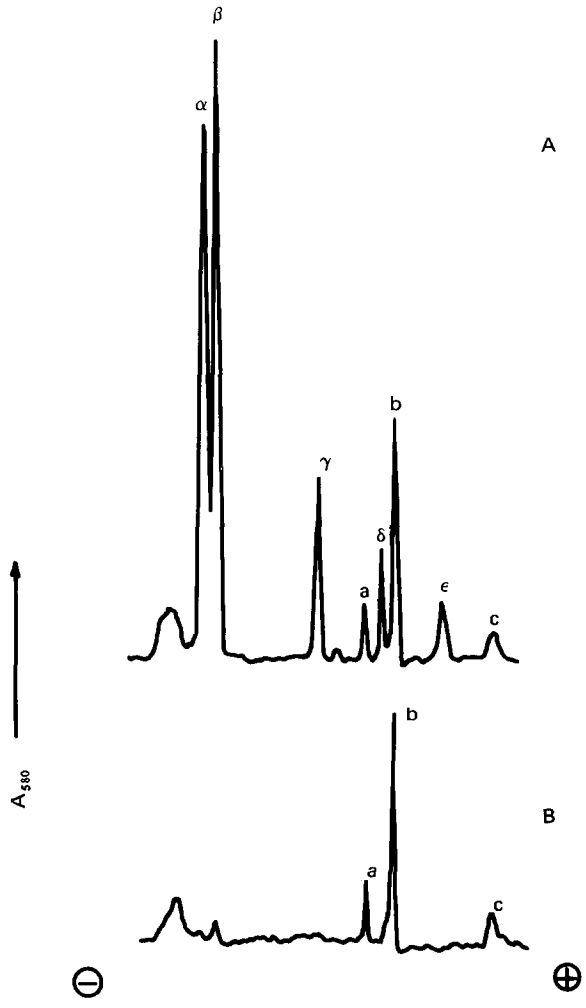

Fig.1. Polypeptides of (A) the F_1F_0- and (B) the F_0-preparation. The enzyme preparations (15 μg F_1F_0, 4 μg F_0) were subjected to SDS gel electrophoresis as in section 2. Protein was stained with Coomassie blue R-250 and the absorbance at 580 nm was recorded. Greek letters denote F_1 subunits; app. M_r of F_0-subunits a (24 000), b (19 000), c (8500).

of deoxycholate*. The pretreated enzyme was dissociated by incubation with KSCN and EDTA in the cold and the aggregated F_0 separated by ultracentrifugation.

Fig.1 shows the subunit composition of the F_0 preparation in comparison to the original F_1F_0 preparation. SDS gel electrophoresis reveals residual amounts of the β-subunit of F_1 and the polypeptide contamination of app. M_r 66 000. The predominant polypeptides of app. M_r 24 000, 19 000 and 8500 are the subunits of F_0 (a,b,c).

F_0 and phospholipids were reconstituted to proteoliposomes and tested for H^+-conduction. Vesicles were loaded with K_2SO_4; addition of valinomycin catalyzed an electrogenic K^+-efflux which caused an equivalent H^+-influx assayed as quenching of acridine dye fluorescence or by a pH-electrode. In agreement with [16,18] both methods lead to equivalent results. The specific H^+-conduction of F_0-proteoliposomes is 20–40 X enriched compared to the activity of membranes (table 1). The H^+-conduction of F_0-proteoliposomes can be greatly inhibited by treatment with DCCD or by addition of F_1. The undissociated F_1F_0 complex shows only residual H^+-conducting activity after reconstitution of proteoliposomes. The specificity of F_1 binding to isolated F_0 was demonstrated by reconstitution of ATP-dependent H^+-translocation assayed as quenching of acridine-dye fluorescence (table 2).

Table 1
H^+-conduction (influx) of proteoliposomes

Reconstituted fraction	Fluorescence test (E_{fl}/mg)	pH-electrode (nmol $H^+ \cdot min^{-1} \cdot mg^{-1}$)
F_0	2565	870
+80 μM DCCD	24	2
$+F_1$ [a]	151	41
F_1F_0	16	5
F_1-depleted membranes	65	44

[a] Blocking of F_0 by F_1 was brought about by adding 10 μg F_1/μg F_0 to the sample before dialysis

Preparation of proteoliposomes, loading of proteoliposomes and membrane vesicles with K^+ and assay of H^+-influx were performed as described in section 2

Table 2
ATP-dependent H^+-translocation of proteoliposomes

Reconstituted fraction	Fluorescence test (E_{fl}/mg)
F_0	<1
$F_0 + F_1$ [a]	1910
F_1F_0	2332
Membranes	130

[a] F_1 (50 μg) was incubated with F_0-proteoliposomes (max. 5 μg protein) in 1 ml test buffer for 5 min at room temperature – Addition of the uncoupler, 20 μM TTFB, to the test buffer decreased the activity below 1 E_{fl}/mg in all tests

Preparation of proteoliposomes and membrane vesicles as well as fluorescence test of ATP-dependent H^+-translocation were performed as described in section 2

4. Discussion

A method for the preparation of F_0 from F_1F_0 has been described for the ATP-synthase of the thermophilic bacterium PS 3 [18] and for *E. coli* [23,24].

The F_0 preparation in [23] was obtained by washing F_1F_0 proteoliposomes with low ionic strength buffer to remove F_1. Considerable amounts of F_1 subunits are still associated with the washed proteoliposomes probably because of unspecific adsorption [21]. The partially purified F_0 preparation shows a high, DCCD-sensitive activity in H^+-conduction. However, it was not tested whether H^+-conduction can be blocked by F_1 or if F_1F_0-activities can be reconstituted with F_1.

The F_0 preparation of PS 3 [18] was prepared by treating F_1F_0 with 7 M urea. The pure F_0 is active in H^+-conduction and F_1F_0 activities can be reconstituted upon addition of F_1. But the quantitative interpretation of their data led then to the conclusion that only 15% of the F_0 survived the drastic urea treatment fully functional, the rest of F_0 being partially denatured [18].

The F_0 preparation in [24] was also obtained by urea treatment of F_1F_0. A comparison of the H^+-conducting activity with membranes or other F_0 preparations is not possible because no quantitative measurement of H^+-conduction has been performed and it is not known how much protein was used for the test.

* We assume that this procedure also removed most of the Aminoxid WS 35, because after dissociation of F_1F_0 in the presence of this detergent F_0 could not be separated by a simple ultracentrifugation

Several lines of evidence make it likely that only a residual activity was measured: (i) The extremely stable proteins [25] of the thermophilic bacterium PS 3 were considerably denatured by this treatment; (ii) In the course of our experiments we found that after treatment of F_1F_0 with >2.5 M urea, ATP-dependent H^+-translocation of F_0 proteoliposomes could not be reconstituted by addition of F_1; >4 M urea resulted in a F_0 preparation which after reconstitution of proteoliposomes showed <10% of the specific H^+-conduction of native F_0; (iii) In contrast to the other F_0 preparations ([18,23], this paper) the proportion of the F_0 subunits is greatly altered compared to the original F_1F_0, subunit a is missing and 75% of subunit b had been removed as judged by comparison to subunit c. In *E. coli* membranes both subunits, a and b, are necessary for the expression of H^+-conduction [14]. The ability of F_0 to reconstitute F_1F_0-activities with added F_1 was not tested.

Treatment of F_1F_0 by 1 M KSCN allows the isolation of a F_0 preparation which is essentially free of F_1 subunits. Comparison with the original F_1F_0 preparation shows that the proportion of the 3 F_0 subunits (a,b,c) is not altered. Two important activities could be reconstituted with this preparation: H^+-conduction via F_0 and ATP-dependent H^+-translocation via F_1F_0. The H^+-conduction of F_0 proteoliposomes is highly sensitive to DCCD and can be strongly suppressed by addition of F_1. This excludes an unspecific H^+-conduction by integration of a denatured protein fraction. The blocking of the H^+-conductor by F_1 rests on the proper binding of F_1 to F_0 as could be shown by the reconstitution of ATP-dependent H^+-translocation. The reconstituted ATP-dependent specific activity of proteoliposomes is 82% compared to the ATP-dependent specific activity of proteoliposomes containing untreated F_1F_0. In agreement to this F_1 suppresses F_0-dependent H^+-conduction to 94%. Thus it seems that mainly a functional F_0 was isolated.

The method of electro-impelled H^+-flux in proteoliposomes seems to be not suitable for a detailed enzymological study on F_0, i.e., pH-optimum, voltage dependence, ion selectivity, kinetics. Investigations using F_0 inserted into black lipid films are underway.

Further biochemical, immunological and genetical characterization of native membranes and purified F_0 are necessary to elucidate the function of the different F_0 subunits for the various biological activities of F_0.

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